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IMPROVEMENT OF ALFALFA PRODUCTIVITY THROUGH THE DEVELOPMENT OF "TANNIN ALFALFA"

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THE SASKATCHEWAN AGRICULTURE DEVELOPMENT FUND

Re:

Project #98000102

Title: Improvement of Alfalfa Productivity Through the Development of "Tannin Alfalfa"

Prepared and submitted April 26, 2001 by

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Abstract/Summary

Genes which appear to regulate tannin accumulation in plants were isolated and characterized using molecular techniques. New alfalfa germplasm was also developed by inserting genes that raised flavonoid gene expression in leaves. Several genotypes responded to natural daylight and cold conditions by strongly increasing tannin-related compounds called anthocyanins. This material can be used by molecular plant breeders as elite recipient germplasm for additional genes required to develop bloat-safe alfalfa with leaf tannins.

Executive Summary

Bloat is a digestive disorder that reduces intake and can eventually cause death in cattle grazing on rapidly digested forage such as alfalfa. A moderate tannin content in fodder prevents bloat in grazing cattle. Tannins also improve protein utilization in cattle and sheep, by protecting feed protein that is normally lost as urea by the rumen (15-25%), so that it can be taken up by the intestine (=bypass protein). Tannins inhibit intestinal parasites. Tannins and a variety of flavonoids also inhibit plant diseases and insect pests.

The development of alfalfa cultivars which contain leaf tannins would provide a high-return grazing option that requires much less management than existing cultivars and is bloat safe. The availability of tannin-containing cultivars would stimulate an expansion (estimated at 60%) of the cow-calf herd, diversify farms, strengthen Saskatchewan's competitive position as a supplier to Alberta and US feedlots, and stimulate growth of local feedlots. Improvement in hay quality could also stimulate an expansion in the backgrounding industry. Sileage made from "Tannin Alfalfa" will be less prone to spoil, because tannins inhibit spoilage bacteria. Eventually, "Tannin Alfalfa" will become the most desirable forage for seeded pasture, with a positive impact on the Forage Seed Industry.

In this project, genes which appear to regulate tannin accumulation in plants were isolated and characterized using molecular biology techniques. Two provisional US patent applications were submitted to protect these genes and provide AAFC and Saskatchewan with an intellectual property position in this fast-moving area of research. The remaining required data is being collected for full submission in 2001.

New alfalfa germplasm was also developed by inserting genes that raised flavonoid gene expression in leaves. While most of these genotypes are not visibly different from non-transgenic alfalfa, several responded to natural daylight and cold conditions by strongly increasing anthocyanin content in leaves and stems. Anthocyanins are coloured flavonoid compounds close in structure to condensed tannins. One of these latter genotypes will be ready to offer to molecular plant breeders after suitable phytochemical analysis of field-grown material and submission of a third patent application. This genotype can be termed "Phase 1" material, since it will serve as recipient germplasm for the additional genes we believe will be necessary for the development of Tannin Alfalfa".

All the transgenic alfalfa will be evaluated for gene expression and phytochemical patterns in a field trial we are developing under western prairie conditions. As well, the transgenics will be tested for disease resistance and overwintering ability. Subsequent to this ADF report, other unique phenotypes will be recovered and will serve as additional germplasm to develop tannin alfalfa.

In addition to these achievements, one invited scientific book chapter and two scientific posters were developed on the findings. Several scientific publications will be submitted after the full patent applications are ready.

106-01166/

Technical Report

Background

Bloat is a digestive disorder that causes distressed breathing, reduces food intake, and can eventually cause death in cattle grazing on rapidly digested forage such as alfalfa[1]. A moderate tannin content in fodder prevents bloat in grazing cattle. Tannins also improve protein utilization in cattle and sheep, by protecting feed protein that is normally lost as urea by the rumen (15-25%), so that it can be taken by the intestine (=bypass protein)[2]. Tannins inhibit intestinal parasites. Tannins and a variety of flavonoids also inhibit plant diseases and insect pests.

The development of alfalfa cultivars which contain leaf tannins would provide a high-return grazing option that requires much less management than existing cultivars and is bloat safe. The availability of tannin-containing cultivars would stimulate an expansion (estimated at 60%) of the cow-calf herd, diversify farms, strengthen Saskatchewan's competitive position as a supplier to Alberta and US feedlots, and stimulate growth of local feedlots. Improvement in hay quality could also stimulate an expansion in the backgrounding industry. Sileage made from "Tannin Alfalfa" will be less prone to spoil, because tannins inhibit spoilage bacteria. Eventually, "Tannin Alfalfa" will become the most desirable forage for seeded pasture, with a positive impact on the Forage Seed Industry.

Scientific Strategy: How to Achieve Tannin Expression in Alfalfa Leaves

Tannins are large plant phenolic compounds which are structurally related to the small phenolic compounds (anthocyanins) that cause purple colours in alfalfa flowers (Fig. 1). Alfalfa accumulates tannins only in seedcoats[3], although several other forage legumes (sainfoin, trefoils) also produce tannins in leaves and flowers. Alfalfa leaves normally only produce other small, related compounds called flavone and isoflavonoid glycosides ([4,5] and Gruber et al., unpublished). These different classes of compounds, i.e., tannins, anthocyanins and flavones, are synthesized in plants using one large set of common flavonoid genes, followed by genes unique to each branch of the flavonoid pathway (Fig. 1). The lack of tannins in alfalfa leaves reflects the fact that, in our hands, chalcone synthase (CHS) is moderately expressed and dihydroflavonol reductase (DFR), flavanone *3B*-hydroxylase (F3H), and leucocyanidin reductase (LCR) genes are poorly expressed (Fig. 1). Other laboratories find that CHS is strongly expressed during a disease attack and that DFR is moderately expressed and F3H is poorly expressed under healthy conditions[6,7,8].

To date, it has not been possible to develop alfalfa germplasm with forage tannins through direct plant selection and mutation[9]. Recently, a patent application by Pioneer Hi-Bred proposed a method in which germplasm was selected with a higher density of leaf hairs than usual[10]. The hairs contained low levels of condensed tannins, but not enough to develop bloat-safe germplasm. Additionally, plant transformation with anthocyanin regulatory genes was used to induce higher levels of tannins in birdsfoot trefoil, as well as to induce white clover leaves to produce the tannin-related coloured compounds, anthocyanins [11-13]. In the ADF project, the latter concept was applied towards developing "Tannin Alfalfa", recognizing that alfalfa would require its own unique blend of genes to produce leaf tannins.

Objectives

The objective was to isolate and develop tannin- and anthocyanin-related genes into plant transformation vectors, and to transfer them into alfalfa to stimulate leaf gene expression in favour of condensed tannins or flavonoids closely related in structure.

Methods

Development and Analysis of Elite Transgenic Alfalfa Genotypes:
Five flavonoid biosynthetic or regulatory transgenes from maize, alfalfa and petunia (*CHS*[7,14], *F3H*[6], *B-Peru*[15], *C1*[16], *Lc*[17]) were used in the project. Some of these genes had been introduced into alfalfa already through funding from AAFC Saskatoon and a previous grant to Dr. Gruber from the Alberta Cattle Commission. The genes were either received from other laboratories or cloned by PCR using sequence data available through the scientific literature. Figure 1 illustrates the place of these genes in the flavonoid biosynthetic pathway.

Genes were developed into transformation vectors using a single copy 35S promoter, an enhanced promoter containing 2 copies of 35S and an enhancer [(35Sx2)-AMV], or promoter from the pea small subunit of ribulose bisphosphate carboxylase [ssRBC]. Both promoters were gifts of the Plant Biotechnology Institute in Saskatoon. Vectors were inserted into alfalfa by *Agrobacterium*-mediated plant transformation[18]. Plants selected by kanamycin resistance were tested by Southern and Northern hybridization to determine whether native flavonoid genes that are poorly expressed or absent in alfalfa leaves were expressed. DNA and RNA were isolated from tissue frozen in liquid nitrogen immediately after harvest. RNA was separated on formaldehyde agarose gels, and DNA was separated on gels containing Tris-acetate buffer. Nucleic acids were denatured, blotted onto nylon membranes, and hybridized to gene probes labelled with ^{32}P-dCTP by random priming. Clones were selected as suitable candidates for additional experimentation if the transgene showed moderate to high expression on Northern blots in leaves and/or a visible phenotype.

Transgene expression and phenotypic analysis (presence of anthocyanins, hairs and *F3H* gene expression) was conducted on leaves harvested from genotypes containing *CHS*, *B-Peru* or *C1* during growth in the field at University of Guelph. Subsequently, all transgenic genotypes were transported to the Saskatoon Research Centre, cloned by rooting cuttings, and observed under a range of conditions in two greenhouses, a growth chamber, or in pots placed during the day in the Saskatoon Centre farm yard. Clones containing an *F3H* or *Lc* transgene were not evaluated in the eastern field trials since they were not mature enough at the time of the trial.

Isolation and Characterization of Novel Tannin Genes:
Novel legume regulatory gene sequences were obtained from leaf tannin-accumulating big *trefoil (Lotus uliginosis)* by amplifying and sequencing genomic PCR products using primers homologous to the maize anthocyanin-regulatory genes, *delila* and *B-Peru*. The barley regulatory gene was initially isolated as two small PCR sequences and a lambda barley genomic DNA clone by Dr. Jon Falk, a visiting fellow working with Dr. Gruber at The Carlsberg Laboratory, Denmark. Dr. Heather Ray completed most of the work on this gene in Saskatoon after Dr. Falk obtained a new position in Germany. The fragments were isolated by Representational Difference Analysis of cDNA from developing seedcoat dissected from *ant*13 tannin-free and wildtype tannin-accumulating barley lines. In *ant*13, transcription of the major flavonoid genes (CHS, F3H, DFR) was suppressed and activity of the major tannin branch enzyme (LCR) was absent[19]. These fragments were used by Dr. Ray to obtain additional barley genomic DNA clones and larger genomic PCR fragments to piece together the sequence data. In addition, 3'- or 5'-RACE was used to get larger cDNA fragments. Sequencing was contracted through the Plant Biotechnology Institute, Saskatoon. Sequence data was analyzed by LaserGene™ and by submission to BLAST. Transient expression was conducted in barley seedcoats dissected from *ant*13 tannin-free lines and from wild type barley using a biolistic gun in collaboration with the Plant Biotechnology Institute, Saskatoon.

Lysed bacterial cells from the *banyuls* cDNA clone were evaluated for leucocyanidin reductase enzyme activity using a Hewlett Packard 1050 HPLC fitted with a Novapak C18 column and a Berthold radio-HPLC detector using a methanol-acetic acid gradient[19]. ^{3}H-*cis*-leucocyanidin substrate for the enzyme assay was synthesized in the laboratory.

Results

Development of Elite Transgenic Alfalfa Genotypes:
Table 1 illustrates the progress to select elite genotypes from the 187 transgenic plants developed through the joint ADF-funded project. Evaluation of Guelph field-grown material containing the *CHS*, *B-Peru* or *C1* transgene indicated several genotypes per construct that accumulate a range of transgene mRNA in young and mature leaf tissue (Table 1). Several of the sense and antisense *C1*-transgenic genotypes also showed moderate accumulation of *F3H* gene transcripts (Table 1). Expression of the *F3H* structural gene is normally completely blocked in leaves of non-transformed control plants. Testing the expression of flavonoid structural gene expression in other genotypes is not complete at this time.

After fall temperatures dropped several times below 0° C in December, 1999, 21 genotypes from *CHS*, *B-Peru* and *C1* field transgenics produced a deep, red colour in their leaves. This seasonal phenotype did not seem to be transgene-specific and was not investigated.

Among two populations of *Lc*-transgenic genotypes tested in the laboratory, we observed several genotypes with strong transgene expression (Table 1). We also observed clones with a red-purple anthocyanin leaf phenotype newly established under warm, intense light conditions in spring in the University of Guelph greenhouse (Table 1, Fig. 2). Anthocyanins are red-purple flavonoid endproducts that have several biosynthetic steps in common with condensed tannins. Several of these phenotypes also had an increased number of leaf hairs in young green shoots. However, the anthocyanin phenotypes were completely suppressed when plants were transferred to the AAFC Saskatoon Centre city greenhouse, and only weakly expressed in one genotype (A01-088-19) when grown under higher light intensity at the Saskatoon Centre farm greenhouse. A strong red leaf and stem anthocyanin phenotype was observed when A01-088-19 was grown in a growth cabinet at 4° C under lower intensity fluorescent lighting (200 *u*E) or at room temperature under higher fluorescent light intensity supplemented with incandescent bulbs (440 *u*E), or when the pots were placed under natural conditions in the Saskatoon farm yard (1700-2000 *u*E, 16-22° C, wind) (Table 1, Fig. 2). Red coloration may be visible within 24 h of placing A01-088-19 under inducing conditions. Control non-transgenic plants or non-*Lc*-expressing transgenic plants did not display this phenotype. Several other *Lc*-transgenic genotypes (A01-088-1,4,19) with strong transgene expression patterns also developed a strong red phenotype within 24 h of being placed under natural conditions. The phenotype was also recovered in several genotypes within the 2nd Lc-transgenic population (series A01-090) under natural light and cold temperature, but the coloration was not as intense under natural conditions as series A01-088.

Flavonoid gene expression was investigated in detail in relation to anthocyanin accumulation, and reported here for genotype A01-088-19. Leaves of A01-088-19 clones growing under very low fluorescent light (<40 *u*E) at 4° C accumulated additional *Lc* and *F3H* transcripts relative to A01-088-19 clones placed in the GH at 20° C (Table 2), in spite of the fact that *Lc* expression was directed by the constitutive 35S promoter. In contrast, relatively fewer *CHS* and *DFR* transcripts accumulated in this genotype under these cold and light conditions. When the light intensity was increased to 200 *u*E at 4° C, anthocyanins visibly accumulated and *Lc*, *F3H* and *DFR* transcripts correspondingly increased several-fold relative to clones at 20° C (Table 2). *CHS* transcript level did not change to any extent in the cold. Similar experiments are underway to characterize flavonoid structural gene expression under natural conditions in all the Lc transgenic plants.

Isolation and characterization of novel tannin genes:
Over the course of the ADF grant, we identified the world's first two genes which appear to specifically regulate flavonoid genes to produce condensed tannins. These are *barmyc* from barley and *legume myc* from *Lotus uliginosis*. These two genes contain the *myc*-like basic helix-loop-helix motif. *Barmyc* differs but legume *myc* is similar in sequence to the anthocyanin-stimulating *myc*-like genes, *Lc* and *B-Peru*. The gene sequences have been submitted as US provisional patent applications. We have also cloned and evaluated sequence data and enzyme activity (in one case) for three candidates for the leucoanthocyanidin reductase

(LAR) gene. LAR is the first class of structural genes in the tannin-specific branch of the flavonoid pathway and includes the LCR gene not expressed in alfalfa leaves. So far, we are not confident that we have the correct gene, although these experiments are still in progress.

Discussion of Results

The accumulation of anthocyanin pigments under a variety of conditions in *Lc*-transgenic alfalfa and the associated induction of flavonoid gene expression indicates that we were successful at raising overall flux through the flavonoid pathway using the *Lc* transgene. Consequently, we have submitted a provisional US patent outlining the use of *Lc* in developing recipient background germplasm for additional rounds of plant transformation in a method to develop "Tannin Alfalfa". In this method, additional structural genes will be required for the *Lc*-germplasm to divert the anthocyanins into condensed tannins.

The increase in the proportion of *Lc* transcripts under cold conditions and low light compared with the warmer temperature and higher light intensity of the greenhouse, as well as the additional accumulation of transcripts when the light intensity was raised under the cold condition, suggests that the 35S promoter may be responsive to one or more cold induced genes in alfalfa. However, a more likely explanation is that the *Lc* RNA is rapidly degraded when anthocyanins are not needed to protect the plant under the more moderate conditions of the greenhouse. This latter hypothesis is supported by the fact that anthocyanins are strongly accumulated when the *Lc*-transgenic plants are grown under the more extreme conditions of natural daylight. We expect that *Lc* and flavonoid structural gene transcripts will also accumulate coordinately with the strong anthocyanin accumulation observed under natural light.

Anthocyanin and gene expression profiles documented under a rhythm of natural light and temperature conditions will be strong indicators of the expression pattern we can expect in the field for the Lc-transgenic plants. They may also forecast the pattern of tannin expression when additional tannin structural genes are transferred into these plants.

Flavonoid gene expression patterns have not yet been fully documented for all the transgenic alfalfa genotypes. Documentation will continue over 2001 in field trials at our Saskatoon farm to determine whether we have recovered additional genotypes that will enable us to develop tannins in alfalfa leaves by several different methods.

In the course of the ADF grant, we were unsure whether any of the maize anthocyanin regulatory genes would be able to direct the synthesis of leaf tannins without additional tannin structural genes. Consequently, we also isolated tannin regulatory genes which we expect will likely stimulate the development of tannins in alfalfa directly. At the time of this report, experiments to complete the cDNA sequence for these genes and to characterize differences between a normal gene sequence and the gene present in a mutant plant with no tannin, are in progess to complete patent requirements. The cDNAs will then be tested for their ability to alter tannin expression by introducing them into their germplasm of origina as well as alfalfa. We have preliminary transient expression data suggesting that a 4.5 kb *barmyc* fragment can stimulate tannin synthesis when introduced into the seedcoat of the *ant*13 tannin-free mutant using a biolistic gun, but this data needs additional confirmation.

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Conclusions

Several genes which appear to regulate tannin accumulation in plants have been isolated and characterized. Several new alfalfa genotypes, in which flavonoid gene expression has been raised in leaves, have also been developed.

Indications for further development

We are currently in the process of developing a field trial in Saskatoon to test our laboratory results under harsh western prairie conditions and to determine persistence over the winter. Experiments to determine additional patterns of structural genes in the CHS, F3H, B-Peru and C1 transgenic plants are outstanding and will also be documented by AAFC staff using this field trial material. We have applied for additional funding from ADF to support a technician or postdoctoral fellow to determine detailed flavonoid composition in these field-grown transgenic plants. Plants will also be tested for disease susceptibility and resistance by AAFC staff, since a variety of flavonoid compounds have been associated with pest resistance.

Suitable transgenic genotypes with meaningful gene expression data are also being cross-pollinated by AAFC staff to western-adapted alfalfa varieties, AC Grazeland and Peace. These experiments will enable us tto assess the effect of the transgenes on fertility, seed production and seed tannin gene expression. These pollination experiments also begin the process of pyramiding several transgenes within the same line. These experiments are expected to improve the capacity of alfalfa to stimulate the flavonoid pathway in a stable fashion, to provide additional methods to develop Tannin Alfalfa, and to increase the chance for survival under western prairie conditions.

Since we were successful at matching the ADF grant with funds from AARI for the third consecutive year, Dr. Heather Ray will continue in her present role to develop the regulatory and structural genes outlined in this report as additional molecular tools to develop Tannin Alfalfa until at least March 31, 2002.

Information of benefit to producers, processors, or government

We have isolated several genes which appear to regulate tannin accumulation in plants. When the cDNA sequences are deposited and full patent applications submitted, we will be able to offer these genes to other molecular plant breeders also interested to develop Tannin Alfalfa. At the same time, we have developed transgenic alfalfa genotypes in which flavonoid expression has been stimulated and resulted in the accumulation of anthocyanins in leaves. This plant material can be used as recipient germplasm for the insertion of structural genes to re-direct the leaves to accumulate tannins rather than anthocyanins. This germplasm could also be offered to plant breeders interested to develop Tannin Alfalfa.

Project Personnel

Dr. Margaret Gruber was the overall project leader and directed the project at AAFC Saskatoon. Dr. Brian McKersie directed plant transformation experiments at University of Guelph until Sept. 1999 when he left to assume a position at BASF (USA). Dr. Steve Bowley conducted the field trial at University of Guelph sites over the course of the project and assumed Dr. Brian McKersie's role as supervisor of the transformation experiments in Sept. 1999. Dr. Bruce Coulman provided assistance and advice with the plant fertility and Saskatoon field trial experiments.

Dr. Heather Ray salary was paid by a combination of ADF and AARI throughout the project. Ms. Ning Chen was mainly funded by U. of Guelph until March 2000, with $14,000 in total funded by ADF/AARI. AAFC Saskatoon funded Ms. Trish Auser from 1998-July, 1999, Ms. Laureen Blahut-Beatty from Aug. 1999-Aug. 2000, Mr. Stefane Dupont from Sept. 1999-March, 2001, and Ms. Terry Huebert from Jan. 2001-March, 2001. Salaries are outlined in the attached financial statement.

Project-developed materials

1. Transgenic alfalfa germplasm ready for disease and persistance testing and detailed phytochemical analysis under western prairie conditions. Some of the germplasm is ready to receive additional structural genes required to develop Tannin Alfalfa.
2. Genes to regulate condensed tannin biosynthesis in plants.

3. 3 provisional USA patent applications, 1 invited scientific book chapter, 1 scientific poster (see restrictions on publications and acknowledgements below).

Meetings to promote industry investment

Reorganization issues prevented the completion of any industry investment agreements during the course of the ADF grant, but discussions are still ongoing with the most desirable of two major forage breeding companies that were interested to invest in the project. Dr. Gruber will be meeting with this company and its partners in July 2001. The name of the company and partners is confidential at this time.

Publications on Tannin Alfalfa since Project Initiation

NB: Pre-2000 presentations and articles do not include work conducted by ADF. These include related work supported earlier by AAFC, the Alberta Cattle Commission and The Carlsberg Laboratory. They are included because they were published during the period of the ADF grant and involve the Tannin Alfalfa project.

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Due to the need for confidentiality while developing an intellectual property portfolio for Tannin Alfalfa over the last few years, we did not present any of the work conducted through the ADF grant to either the forage and cattle-producing or scientific community until provisional patent applications were submitted in 2000. Pre-2000 presentations and articles reported above only included work conducted and previously funded by AAFC, the Alberta Cattle Commission and the Carlsberg Laboratory on condensed tannin biosynthesis. Since 1998, ADF support was acknowledged in detailed confidential annual reports to the Alberta Agriculture Research Institute (Forage Committee), a joint supporter of the development of Tannin Alfalfa. Once provisional patent applications were submitted, support by ADF was acknowedged at an invited keynote oral presentation by Dr. Gruber on the overall "Tannin Alfalfa" project in November, 2000 at the 2nd International Symposium. Molecular Breeding of Forage Crops 2000 in Lorne, Australia (publication #7 above).

In 2001 and 2002, ADF will be acknowledged to the forage and cattle-producing community at presentations on the progress of Tannin Alfalfa planned by Dr. Gruber. As well, ADF support will be acknowledged in scientific publications which will be written after the full patent applications are submitted. ADF will also be acknowledged in oral and poster presentations by Dr. Ray in July 2001 at the joint annual meetings for the Canadian and American Societies for Plant Physiology in Rhode Island, USA (abstract titles #1, 2 above).

Table 1: Characteristics of Selected Transgenic Alfalfa Genotypes on April 25, 2001

Transgene name and orientation	Promoter	# Remaining Genotypes	Genotypes promoter and transgene	Leaf RNA expression pattern (immature/mature)	Additional Phenotypes (immature/mature)	Genotypes in Saskatoon 2001 Field Trial
ND, not determined						
			Genotypes containing a Structural Transgene			
A01-041 anti-sense CHS (petunia) pB69	(35Sx2)-AMV	9	7	#1,2,4,5 strong/strong #8,9,11 weak/weak	#8,15 dwarf	Not included
A01-043 anti-sense CHS (alfalfa) pB114	(35Sx2)-AMV	12	12	All 12 medium/strong Repeating	None visible to date	#1,11
A01-044 sense CHS (alfalfa) pB115	(35Sx2)-AMV	6	6	#1,3,5 medium-strong/strong #6,11,12 strong/weak	None visible to date	#1,3,5,12
A04-091 antisense F3H (alfalfa) pB164	ssRBC	27	Repeating tests	repeating tests	None visible to date	Not included
A01-092 sense F3H (alfalfa) pB165	ssRBC	3	3	#1,2,3 Repeating tests	None visible to date	Not included
A13-092 sense F3H (alfalfa) pB165	ssRBC	5	Repeating tests	#4,10,11,12 Repeating tests	None visible to date	#4,10,12
A01-087 sense F3H (barley)	(35Sx2)-AMV	7	Not determined	Not determined	None visible to date	#3,4,7
A04-087 sense F3H (barley)	(35Sx2)-AMV	1	Not determined	Not determined	None visible to date	#5

ND, not determined

Genotypes containing a Regulatory Transgene

A01-045 sense *B-Peru* (maize) **myc** pB116	35Sx2)-AMV	5	5	#1 strong/strong #3,4 strong/weak #10 weak/weak #14 none/medium	None visible to date	#1,3,4
A01-101 anti-sense *B-Peru* (maize) **myc** p169	ssRBC	5	none	none	None visible to date	Not Included
A04-100 sense *B-Peru* (maize) **myc** pB168	ssRBC	8	6	#7,15,16 ND/medium #1,2,5,14,20 none	None visible to date	#7
A01-088 sense Lc (2.4 kb) (maize) **myc** pBAL69	35S	9	4 +ve 5 unclear (high background)	#1,4,9,19 ND/strong #7,13 ND/medium #2,3,14 ND/none	#1,4,9 red cuttings – not tested in cold #1,4,9,19 slightly red cuttings; very light red stems and leaf tips in GH3; #19 strongly red in GC (4°C + 200 uE/20oC + 300 uE); all 4 genotypes purple after 4 days light, #1,9 purple after 24 h #13 light red after 4 days of natural light	#1,4,7,9,19
A01-090 sense Lc (2.2 kb) (maize) **myc** pAL114	35S	17	12 +ve 5 –ve	#5A,37,39,40 strong/ND #9,19 medium/ND #1-4,5B, 7,8,10,13,23,38 none/ND	#1, red in GH1 #5A,39 red at 4°C/200 uE #5B, 13, 19,38 old leaves red in GH1 #5A, old leaves red with staining, #5A,5B,13 young leaves hairy	#1,5A,5B,8,9,13, 19,37,38,39,40
A01-048 anti-sense C1 (maize) **myb** pB119	(35Sx2)-AMV	8	7	#3 medium/high #4,5,6,10 weak/medium #11 weak/medium-strong #14 weak/weak #17 no +ve Southern	#3 medium/weak F3H #4 none/medium F3H #5 no data #6 weak/medium F3H #10 weak/medium F3H #11,14,17 no F3H expression	#3,6,10,17
A01-047 sense C1 (maize) **myb** pB118	(35Sx2)-AMV	4	4	#6,9,11 weak/medium #12 medium/medium	#6,9,11 medium(extra band)/weak F3H #6 dwarf	#6,9,12
A01-089 sense C1 (maize) **myb** pAL71	35S	19	Repeating tests	Repeating tests	None visible to date	Not included

Table 2. Relative mRNA accumulation in an anthocyanin-accumulating transgenic alfalfa genotype.

A01-088-19			Leaf Transcripts* Accumulated			
Growth Facility and Temperature (°C)	Light Intensity at clone surface (*u*E)	Leaf and Stem Colour Response	*Lc*	*CHS*	*F3H*	*DFR* (one expt only)
Cabinet 4°	<40 *u*E	light red	3.3	0.6	4.4	0.4
Cabinet 4°	200 *u*E	Dark red	8.7	1.2	4.6	4
Greenhouse 20°	35-450 *u*E	Green	1.0	1.0	1.0	1.0

*transcripts were semi-quantified by comparing densities of hybridization signals on exposed film with intensity of ethidium bromide-stained rRNA.

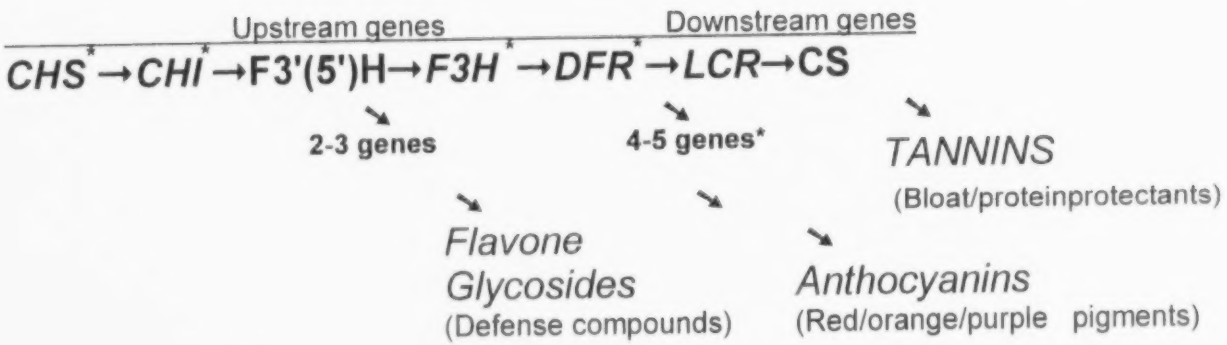

Fig. 1. Biosynthetic pathway leading to anthocyanins, condensed tannins and related phenolics. CHS, chalcone synthase; CHI, chalcone isomerase; F3'(5')H, flavanoid 3'(5') hydroxylase; F3H, flavanone 3β-hydroxylase; DFR, dihydroflavanol reductase; LCR, leucocyanidin reductases; CS, condensing step. *Biochemical genes shown to be activated by anthocyanin regulatory genes, B-Peru, Lc and C1. Other genes illustrated above may also be activated but have not been tested. Genes written in italics are those which are known to be blocked or partly blocked from expressing in alfalfa leaves.

Stimulation of Anthocyanins in *Lc*-transgenic Alfalfa

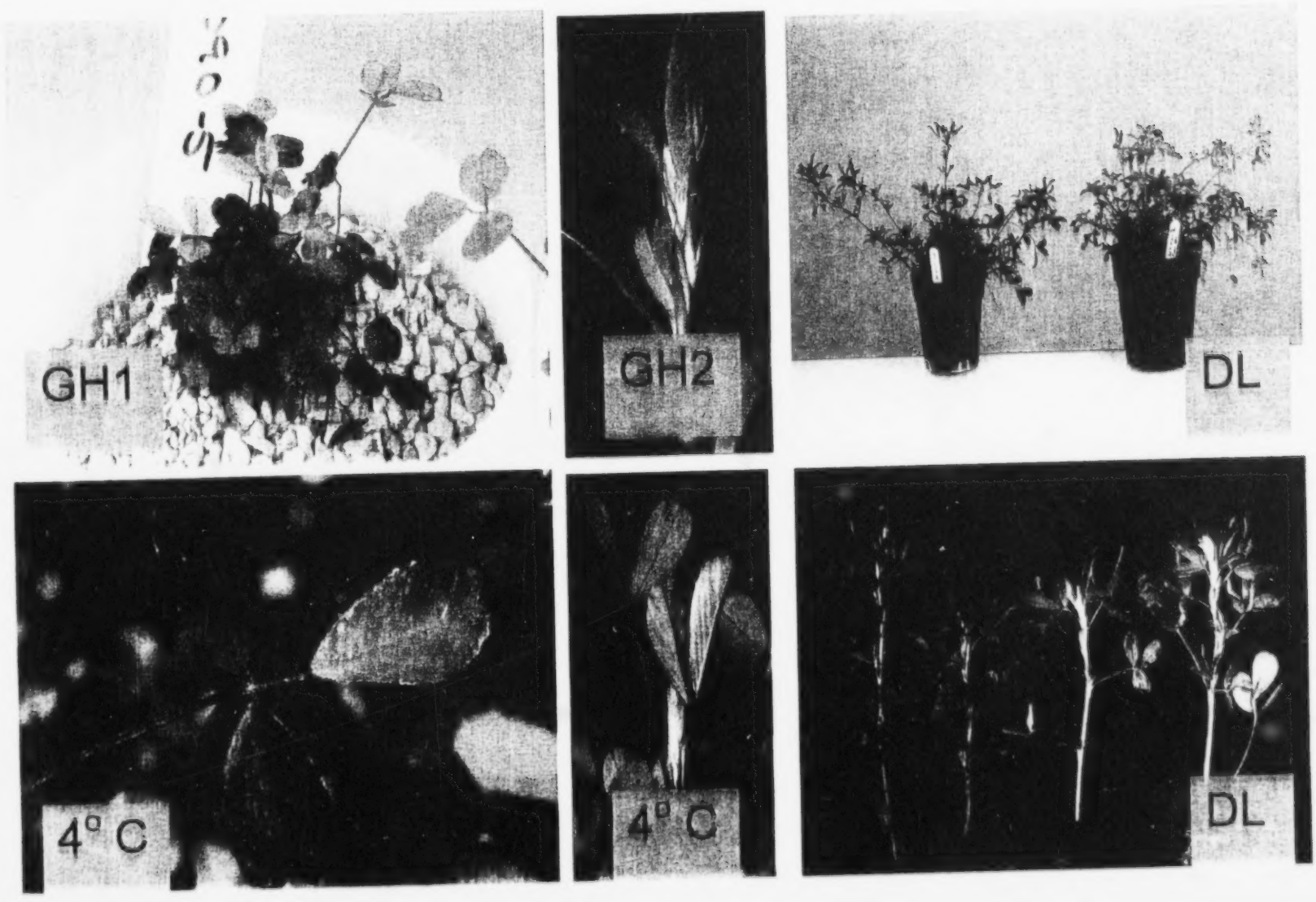

Fig. 2. Accumulation of anthocyanins in Lc-transgenic alfalfa genotypes. Mature leaves from several plants transferred in late March 2000 in the University of Guelph greenhouse at 20° C supplemented with high-pressure sodium lights (GH1), or in a growth cabinet at 4° C under cool-white fluorescent lights (200 uE) (4° C), or in natural conditions in late March in Saskatoon (16-22° C, wind, 1700-1800 *u*E) are dark red with red stems wherever exposed to light (A01-088-19) (DL). Clones developed from rooted cuttings from the same plants do not display this phenotype at 20° C in Saskatoon greenhouse 2 supplemented with high-pressure sodium lights (GH2).